Report

Current Biology

Stomatal Guard Cells Co-opted an Ancient ABA-Dependent Desiccation Survival System to Regulate Stomatal Closure

Highlights

- *Arabidopsis* SLAC1 is activated by ancient drought/ABA-signaling kinases (OSTs)
- Stomata emergence in mosses coincided with SLAC1-type channels activated by OSTs
- SLAC1 evolved motifs to co-opt ancient drought/ABA-signaling pathway via OST1

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In Brief

Fast drought/ABA-signaling in guard cells of higher plants involves the SnRK2 kinase OST1 activating the anion channel SLAC1. Here, Lind et al. show that during evolution of early land plants, SLAC1 co-opted the ancient ABA-signaling pathway by developing motifs at its cytosolic termini that allow ABA-activated OSTs to regulate SLAC1 activity.

Lind et al., 2015, Current Biology *25*, 928–935
March 30, 2015
http://dx.doi.org/10.1016/j.cub.2015.01.067

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Stomatal Guard Cells Co-opted an Ancient ABA-Dependent Desiccation Survival System to Regulate Stomatal Closure

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http://dx.doi.org/10.1016/j.cub.2015.01.067

SUMMARY

During the transition from water to land, plants had to cope with the loss of water through transpiration, the inevitable result of photosynthetic CO_2 fixation on land [1, 2]. Control of transpiration became possible through the development of a new cell type: guard cells, which form stomata. In vascular plants, stomatal regulation is mediated by the stress hormone ABA, which triggers the opening of the SnR kinase OST1-activated anion channel SLAC1 [3, 4]. To understand the evolution of this regulatory circuit, we cloned both ABA-signaling elements, SLAC1 and OST1, from a charophyte alga, a liverwort, and a moss, and functionally analyzed the channel-kinase interactions. We were able to show that the emergence of stomata in the last common ancestor of mosses and vascular plants coincided with the origin of SLAC1-type channels capable of using the ancient ABA drought signaling kinase OST1 for regulation of stomatal closure.

RESULTS

Molecular divergence time estimates suggest that the last common ancestors (LCAs) of land plants diverged into the lineages leading to the paraphyletic group of bryophytes (comprising liverworts [Marchantiophyta], mosses [Bryophyta], and hornworts [Anthocerotophyta]; Figure 1A) and vascular plants about 500 million years ago [6, 7]. Due to temporal variations in water availability, terrestrial plants had to develop mechanisms to survive dehydration and rehydration cycles. While the haploid gametophyte, representing the dominant generation of the poikilohydric bryophytes, lacks dynamic water pores, stomata can be found in the sporophytes of mosses and hornworts [1] (Figure 1A). In contrast, liverworts completely lack stomata [2].

Vascular plants operate paired guard cells that surround the stoma, thus allowing regulation of the stomatal aperture. Inflation of guard cells opens the stoma, whereas deflation results in stomatal closure. When closure is required, K^+ and anions such as chloride and nitrate are released, and the accompanying osmotic water efflux deflates the guard cells and closes the stoma [8]. SLAC1 and SLAH3 from the SLAC/SLAH family shuttle chloride and nitrate across the plasma membrane of guard cells [5]. The activity of these channels is tightly regulated, and the stress hormone abscisic acid (ABA) is a key player in drought-induced stomatal closure [9]. The response of guard cells to ABA has been well documented in the flowering plant *Arabidopsis thaliana* [9]. The signaling pathway consists of a cytosolic RCAR/PYR-type ABA receptor that binds and inactivates the PP2C protein phosphatase ABI1 in the presence of ABA [10] (Figure 1B). This phosphatase in turn controls the protein kinase OST1. In the absence of ABA, ABI1 keeps OST1 dephosphorylated and thus inactive, while, upon binding of ABA to the receptor, the activated receptor binds ABI1, thereby releasing OST1 from inhibition. OST1 activates transcription factors [11, 12] and triggers stomatal closure by phosphorylating SLAC1, which opens this channel and causes anion efflux from the guard cell [3, 4]. Consequently, the membrane potential depolarizes, which activates K^+ release channels. Plants lacking *OST1* show an open stomata phenotype, wilting even upon mild water stress [13, 14].

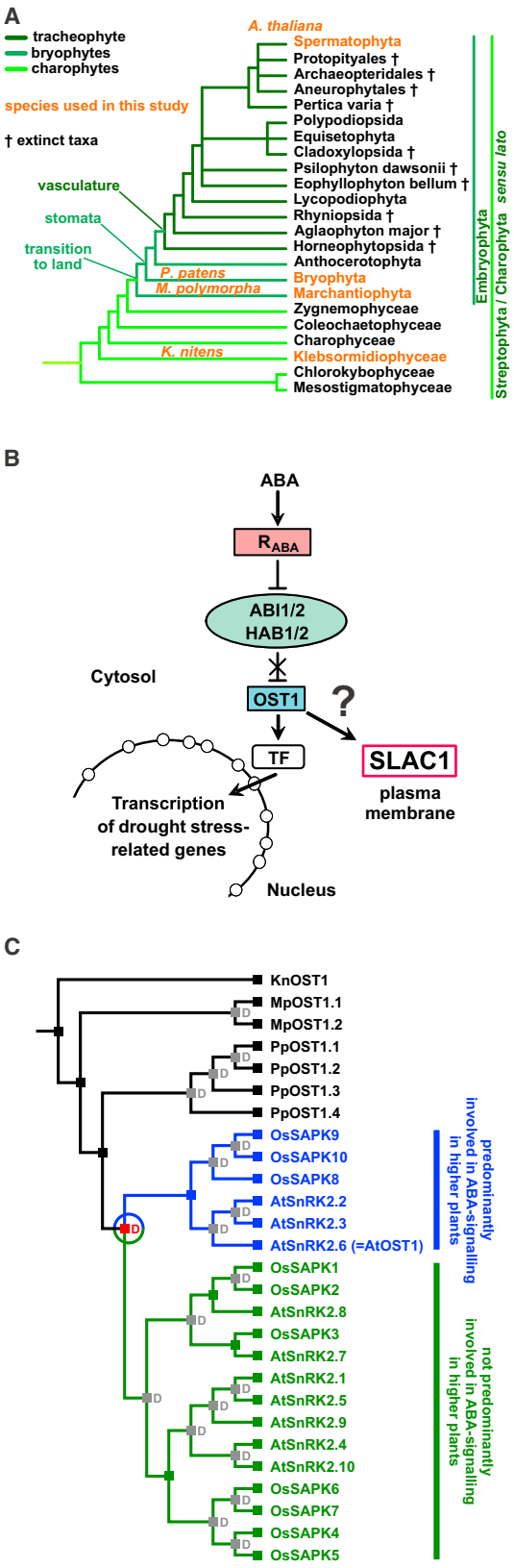

Figure 1. Overview of the ABA-Signaling Pathway and Its Role in Stomatal Closure

(A) Schematic overview of streptophyte evolution.

(B) Schematic representation of ABA signal transduction, including the ABA receptor R_{ABA}, PP2C phosphatases (ABI1/2, HAB1/2), SnRK2 kinases such as OST1, and transcription factors such as ABF2. In *Arabidopsis*, the ABA-dependent regulation of S-type anion channel SLAC1 also uses the ancient ABA-signaling pathway for regulating stomatal closure.

(C) Reconciliation analysis of the phylogenetic relationship between SnRK2-type protein kinases from *Klebsormidium nitens*, *Marchantia polymorpha*, *Physcomitrella patens*, *Oryza sativa*, and *Arabidopsis thaliana*. Phylogenetic analyses were carried out as described [5]. The diversity observed in *Marchantia* and *Physcomitrella* originates from species-dependent duplication events (gray Ds). After the advent of bryophytes, a particular gene duplication event (red D) resulted in the separation of SnRK2s involved in ABA signaling (in *Arabidopsis*: AtOST1, AtSnRK2.2, AtSnRK2.3; blue lines) from those not involved (green lines).

See Figure S1 for a detailed sequence alignment and Table S1 for pairwise identity scores between SnRK2s from different plant species.

OST1 belongs to the plant-specific protein kinase family SnRK2, which is related to the yeast SNF1 kinases [15]. In *Arabidopsis*, the SnRK2 family comprises ten members (SnRK2.1–SnRK2.10; OST1 = SnRK2.6). Three of these, OST1, SnRK2.2, and SnRK2.3, form a separate clade in phylogenetic reconstructions (Figure 1C). Coincidentally, these three kinases are the main players in ABA signaling, as demonstrated by elimination of the fast stomata closure response and slower ABA-dependent processes such as seed germination in a *snrk2.2/2.3/2.6* triple mutant [16–18]. Among these three SnRK2s, however, only OST1/SnRK2.6 is able to regulate the *Arabidopsis* guard cell anion channel AtSLAC1, causing it to open [3].

ABA-signaling elements and transcriptional networks are found to be largely conserved in mosses and liverworts [19–23]. Both the *cis*-regulatory ABA-responsive promoter elements (ABREs) and homologs of transacting factors from the ABF/ABI5 subfamily of bZIP transcription factors binding to ABREs in promoters of ABA-responsive genes have been found in the moss *Physcomitrella* [19, 24–26]. To gain deeper insights into the evolution of fast ABA signaling leading to stomatal closure, we asked the following question: when did early land plants start to use the ancient ABA-dependent drought stress-signaling pathway to control stomatal transpiration (Figure 1B)? The evolutionary process in terrestrial plants toward active stomatal control required the establishment of two prerequisites: (1) OST1 and SLAC1 had to form a functional complex, and (2) both partners had to be specifically expressed in guard cells, but not in the surrounding epidermal cells. Here, we investigated prerequisite (1) in order to determine when SLAC1 was put under the control of the ancient ABA-signaling pathway.

Identification of OST1-like Kinases and SLAC1-like Anion Channels in an Alga, Liverwort, and Moss

As in all mosses, in *Physcomitrella patens*, stomata are found at the base of the spore capsule as part of the sporophyte but are absent from the dominant gametophyte. As described above, liverworts do not have stomata, raising the question as to when molecular mechanisms governing stomatal activity arose. Consequently, we included the liverwort *Marchantia polymorpha* in our studies to test whether OST1-type ABA signaling is functional in both stomata-bearing and non-stomata-bearing

bryophytes. The closest living relatives of land plants (embryophytes) are the charophyte or streptophyte algae. Klebsormidiophyceae is the most basal, multicellular class of the streptophyte algae. The lineage is represented in our study by *Klebsormidium nitens*, a simple filamentous alga that lives in both fresh water and terrestrial habitats [27].

Thus, in this study, we screened the genomes of *Klebsormidium nitens* (H.R., R.R., D.L., and colleagues, unpublished data), the liverwort *Marchantia polymorpha*, and the moss *Physcomitrella patens* [23] for genes predicted to encode SnRK2s and *SLAC1*. In both the *Klebsormidium* and the *Marchantia* genomes, one S-type anion channel gene could be identified (*KnSLAC1* and *MpSLAC1*), while the *Physcomitrella* genome harbors four, two of which, *PpSLAC1* and *PpSLAC2*, are closely related to the *SLAC1* channel genes of vascular plants, while the other two are related to the *SLAH2/3* group [5]. Additionally, we identified one kinase of the SnRK2 type in *Klebsormidium* (KnOST1), two in *Marchantia* (MpOST1.1 and MpOST1.2), and four in *Physcomitrella* (PpOST1.1–PpOST1.4). Interestingly, all seven basal plant SnRK2s share greater similarities with the three ABA-associated *Arabidopsis* kinases, especially with OST1, compared to those not predominantly involved in ABA signaling (Figure S1 and Table S1). Similar to AtOST1, AtSnRK2.2, and AtSnRK2.3, the kinases KnOST1, MpOST1.1, MpOST1.2, and PpOST1.1–PpOST1.4 share a characteristic domain at the C-terminal end that is conserved among ABA-responsive SnRK2s of vascular plants (domain II; [28]). The evolutionarily reconciled relationship of the SnRK2 protein family is shown in Figure 1C. This tree suggests that the LCA of the investigated plants had just one SnRK2 gene. A gene duplication event in the LCA of angiosperms resulted in the separation of two lineages. One of these, the OST1 clade, is associated predominantly with ABA signaling in vascular plants, whereas the other is not [16].

Arabidopsis OST1 Orthologs from *Physcomitrella*, *Marchantia*, and *Klebsormidium* Are Capable of Transducing ABA-Induced Gene Expression

ABA-induced transcription can be monitored by *RD29B* expression, a potent marker for the regulatory activation of dehydrin genes [29–31]. We expressed AtOST1, PpOST1.2, MpOST1.1, and KnOST1 in mesophyll protoplasts of the *Arabidopsis atsnrk2.2/2.3/2.6* triple mutant, which contains a luciferase-based dehydrin reporter system [11]. In this system, *Arabidopsis* OST1 and its orthologs from *Physcomitrella*, *Marchantia*, and *Klebsormidium* restored the ABA-induced *RD29B* expression (Figure 2A). Thus, OSTs from species lacking stomatal complexes, such as liverworts and algae, can already transduce ABA-dependent transcription. This finding is well in line with successful *Arabidopsis ost1* mutant complementation studies using the *Physcomitrella* and *Selaginella* OST1 homologs [32, 33]. It is therefore tempting to speculate that the transcriptional ABA response is much older than the SnRK2-dependent activation of anion channels.

Arabidopsis SLAC1 Is Activated by OSTs from All Tested Plant Species

In an OST1 targeted approach, we aimed to gain new insights into the origin of guard cell ABA signaling by comparing the established *Arabidopsis* SLAC1/OST1 system with the equivalent SLAC1 and OST1 gene products of *Physcomitrella*, *Marchantia*, and *Klebsormidium*. Interestingly, all moss, liverwort, and alga SnRK2s tested were able to activate AtSLAC1 in the *Xenopus laevis* oocyte system (Figure 2B). This activation could be blocked by co-expression with the *Arabidopsis*, moss, or *Marchantia* type-2C phosphatases AtABI1, PpABI1a, PpABI1b, or MpABI1 (Figure 2C), indicating the ability of these kinases to fully replace AtOST1 in the ABA-signaling cascade. Out of all the *Physcomitrella*, *Marchantia*, and *Klebsormidium* SLAC/OST pairs tested, however, only PpOST1.2 elicited anion currents when expressed together with PpSLAC1 (Figures 2B and 3A). The PpOST1.2-activated moss anion channel PpSLAC1 is selective for chloride and nitrate, but not for malate and sulfate anions (Figures 3B and 3C). Furthermore, when the external chloride concentration was increased, the voltage-dependent open probability of PpSLAC1 shifted toward more hyperpolarized potentials (Figure 3D). This indicates that the permeated ion also modulates the activity of the channel, similar to the situation with AtSLAC1 [3]. Additionally, split-YFP studies (bi-molecular fluorescence complementation, BiFC) in oocytes with one-half of the YFP fused to the C terminus of PpSLAC1 and the second half of the YFP fused to the C terminus of different kinases showed, for all the tested OSTs, that they physically interact with PpSLAC1 (Figures 3E and S2A). This indicates that *Physcomitrella* already encodes functional orthologs of the key players AtOST1 and AtSLAC1, in *Arabidopsis* guard cell fast ABA signaling. In contrast to PpSLAC1, however, the second moss S-type channel PpSLAC2, the liverwort MpSLAC1, and the alga KnSLAC1 could not be functionally activated by any of the tested SnRKs (Figure 2B).

Recently, the structure of AtSLAC1 has been modeled on the 3D matrix of an anion channel protein of the bacterium *Haemophilus influenzae* [34]. In the modeled 3D structure, AtSLAC1-Phe^{450} was proposed to function as a gating residue. Consistent with this hypothesis, an F450A mutation transforms SLAC1 into an open, OST1-independent anion channel [34]. To prove that the liverwort and charophyte alga SLACs are expressed in oocytes and to investigate the S-type features of the ancient anion channels, we introduced the above described F-to-A mutation into MpSLAC1 and KnSLAC1. When mutated, both MpSLAC1-F389A and KnSLAC1-F318A elicited anion currents with slow deactivation kinetics reminiscent of the S-type anion currents known from guard cell and *Xenopus* oocyte measurements [3] (Figures S3A and S3D). As expected, the selectivity of the mutant liverwort anion channel was similar to that of seed plant [3] and moss SLAC1 (Figures S3B and S3C; cf. Figure 3B). Interestingly, the *Klebsormidium* SLAC1 was characterized by a stronger nitrate selectivity (Figures S3E and S3F) with a relative permeability ratio $P_{nitrate}/P_{chloride}$ of ~10, while the moss and liverwort SLAC1s showed values of ~3 and ~5, respectively.

The SLAC1/OST1 Module Required for Active Stomatal Closure Is Only Found in Mosses and Vascular Plants

Early members of the Streptophyta sensu lato (Figure 1A), i.e., the Klebsormidiophyceaen algae, liverworts, and mosses, already had SnR type 2 kinases that are all able to activate the *Arabidopsis* AtSLAC1. This demonstrates that they can (1) physically interact with the S-type anion channel and (2) functionally

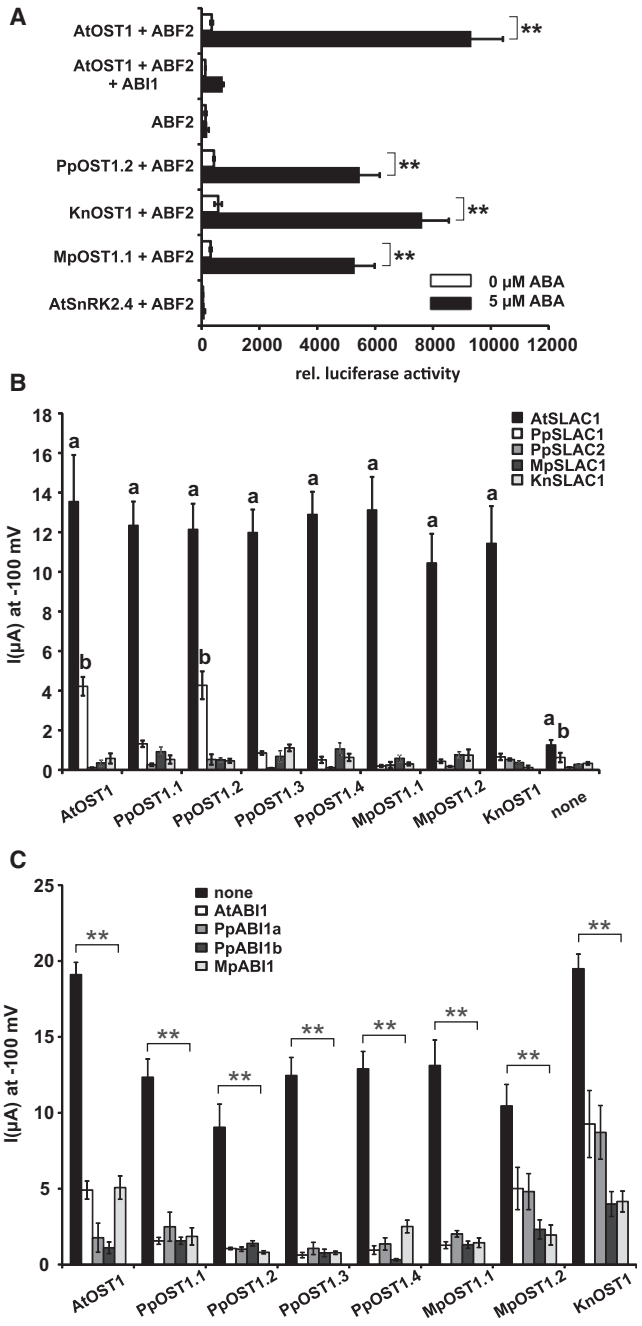

Figure 2. The Transcriptional ABA Response via SnRK2 Kinases Is Older Than the SnRK2-Dependent Activation of Anion Channels

(A) ABA-dependent transcriptional regulation in *Arabidopsis snrk2.2, 2.3, 2.6* triple mutant protoplasts was restored by transformation with any of the tested OST1s. *RD29Bpro-LUC* expression was monitored in *snrk2.2/3/6* triple mutant protoplasts co-transformed with the indicated combinations of *ABI1*, *OST1s*, *AtSnRK2.4*, *ABF2*, and *RD29Bpro-LUC* with or without 5 µM ABA. Data are mean ± SE ($n \geq 6$) from three independent experiments. ANOVA between 0 µM and 5 µM ABA was performed; **$p \leq 0.01$.

(B) *Arabidopsis thaliana*, *Physcomitrella patens*, *Marchantia polymorpha*, and *Klebsormidium nitens* SLAC1-type anion channels and OST1-type SnRK2 kinases were co-expressed in *Xenopus* oocytes in various combinations. Whole-oocyte current measurements in a standard medium revealed that AtSLAC1 is activated by all the tested OST1s. In contrast, PpSLAC1 was active only in the presence of AtOST1 and PpOST1.2. Data show the mean ± SE ($n \geq 4$) of at least two independent oocyte batches. Statistical analyses (ANOVA) compared values obtained for individual SLAC1 anion channels expressed alone with those measured after co-expression with an OST1 kinase; a (AtSLAC1) and b (PpSLAC1) indicate significant differences ($p \leq 0.01$).

(C) AtSLAC1 activation by different OST1s is prevented by moss and liverwort PP2C phosphatases. Instantaneous currents (at −100 mV in standard solution) recorded from oocytes co-expressing AtSLAC1 and OSTs from different species ± *Arabidopsis* AtABI1, *Physcomitrella* PpABI1a/b, or *Marchantia* MpABI1. Data show mean ± SE ($n \geq 8$) from at least two independent oocyte batches. In one-way ANOVA, we compared the values obtained with an AtSLAC1/OST1-kinase pair with those obtained with an AtSLAC1/OST1-kinase/ABI1-phosphatase triplet ($p \leq 0.01$). Note that co-expression of any phosphatase significantly reduced the OST1/AtSLAC1-mediated anion currents (indicated by **).

regulate it. In contrast, none of the OST1s were able to activate any algal, liverwort, or moss S-type channels, with the exception of PpSLAC1. Hence, the differences between PpSLAC1 on the one hand and PpSLAC2, MpSLAC1, and KnSLAC1 on the other might reveal features that are important for fast guard cell ABA signaling (Figure S4A). When comparing the overall structures of the S-type anion channel isoforms tested here, it became apparent that the liverwort and, even more so, the *Klebsormidium* SLAC1 are both characterized by very short cytosolic N termini and C termini, whereas PpSLAC1 and PpSLAC2 have even longer N termini and C termini than AtSLAC1 (Figure S4A). Multiple sequence alignments of the tested SLAC1s with 22 SLAC1s from higher plants (Figure S4A) revealed that the confirmed AtSLAC1 OST1-specific phosphorylation site S120 [3, 35] and the site S59, that is addressed by OST1 and Ca^{2+}-dependent kinases (CPKs) [35] are conserved only in the SLAC1-like channels of vascular plants and *Physcomitrella*. The absence of these sites in KnSLAC1 and MpSLAC1 might therefore explain the OST1-insensitivity of algal and liverwort anion channels. However, the functional differences between AtSLAC1, PpSLAC1 and PpSLAC2 cannot be explained in this way.

To investigate the possible role of the cytosolic N-terminal domain, we replaced the cytosolic N terminus of PpSLAC1 (amino acid [aa] 1 to 215) with the respective domain of AtSLAC1 (aa 1 to 153). As with PpSLAC1, the resulting chimera At_{Nt}-PpSLAC1 also physically interacted with all kinases tested in this study, as visualized by BiFC experiments in *Xenopus* oocytes (Figure S2B). Interestingly, in contrast to PpSLAC1 but in agreement with AtSLAC1, At_{Nt}-PpSLAC1 was activated by all tested OSTs, not just by AtOST1 and PpOST1.2 (Figures 4A and 4B). To investigate whether the N-terminal domain of AtSLAC1 is sufficient for anion channel activation, we also performed a domain switch experiment with PpSLAC2, which was not activated by any of the OST1 kinases (Figure 2B). Despite the fact that At_{NT}-PpSLAC2, as PpSLAC1 and PpSLAC2, physically interacted with all the tested kinases in the BiFC experiments (Figures 3E, S2A, S2C, and S2D), anion currents of the chimera were only marginally activated by some OSTs (Figure 4C). We therefore also replaced the cytosolic C terminus of PpSLAC2 by the respective AtSLAC1 C-terminal domain. The resulting chimera, At_{NT}-PpSLAC2-At_{CT}, interacted with (Figure S2E) and was activated by OSTs from all tested species (Figure 4C). This shows that, in addition to the N terminus, the C terminus also contains important sites for functional SLAC1/OST1 interaction.

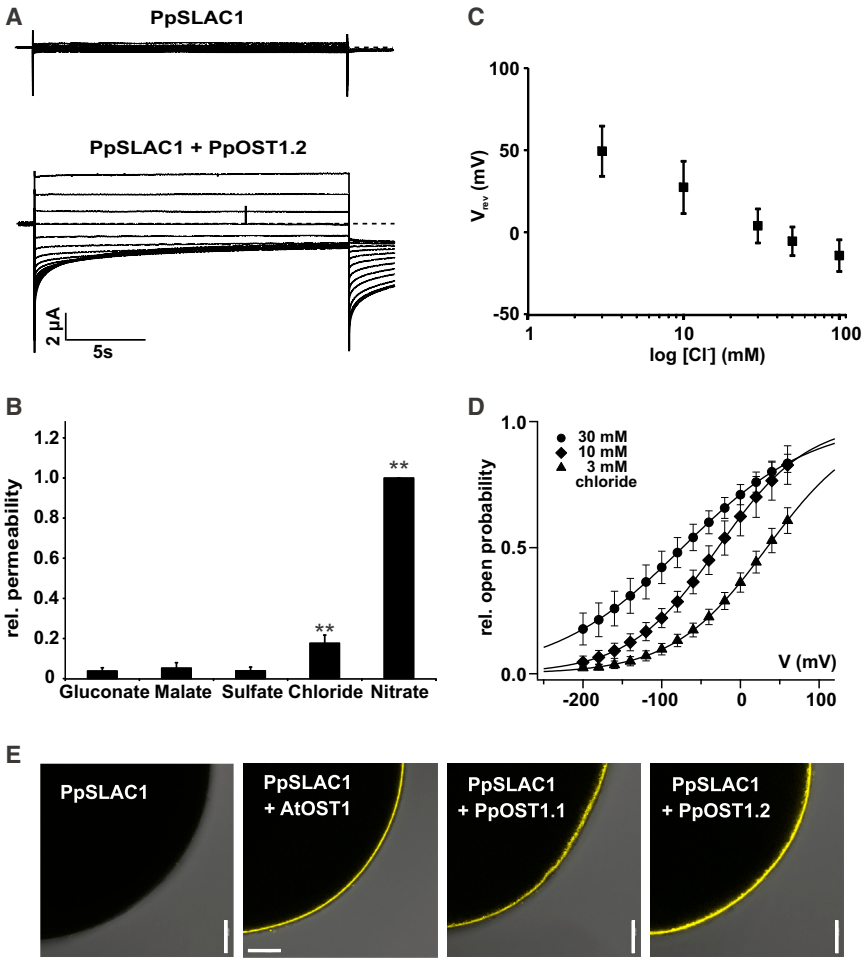

Figure 3. *Arabidopsis* and *Physcomitrella* SLAC1 Are Activated by Conserved SnRK2 Kinases

(A) Example whole-oocyte currents of PpSLAC1, either expressed alone (upper panel) or with PpOST1.2 (lower panel), were recorded in a standard bath solution. Voltage pulses lasting 20 s ranging from +40 to −180 mV in 20-mV decrements were applied (holding potential V_H was 0 mV).

(B) Relative permeability (rel. permeability) of PpSLAC1 co-expressed with PpOST1.2 in *Xenopus* oocytes (permeability for NO_3^- was set to 1). The standard bath solution contained 50 mM of the indicated anion (pH 7.5). Data are mean ± SE ($n \geq 11$) from at least two independent oocyte batches. One-way ANOVA was performed between the values obtained with the impermeable anion gluconate and those measured with the other anions; ** indicate significant differences ($p \leq 0.01$).

(C) Reversal potentials (V_{rev}) of PpSLAC1- and PpOST1.2-expressing oocytes are shown as a function of the logarithmic external chloride concentration ($n \geq 10$; mean ± SE from two independent experiments).

(D) The relative open probability (rel. P_O) of PpSLAC1 activated by PpOST1.2 was calculated at various Cl^- concentrations and plotted against the membrane potential. Data points (mean ± SE; n = 13 from experiments on at least two independent oocyte batches) were fitted with a single Boltzmann equation (solid lines).

(E) BiFC experiments revealed that PpSLAC1 physically interacts not only with the activating kinases AtOST1 and PpOST1.2, but also with PpOST1.1 and all other tested OSTs (see also Figure S2). PpSLAC1:YFP^{CT} was either expressed alone or co-expressed with AtOST1:YFP^{NT}, PpOST1.1:YFP^{NT}, or PpOST1.2:YFP^{NT}. Representative oocytes are shown. Scale bars represent 100 µm.

Closer inspection of SLAC1 proteins from vascular plants revealed a 100% conserved motif of ten aa right after the last transmembrane domain. This motif is also conserved in PpSLAC1, but slightly mutated in PpSLAC2 (Figures 4D, S4A, and S4B). To test whether this conserved C-terminal motif is important for the activation of SLAC-type anion channels, we generated the conserved motif in PpSLAC2 by mutating Q531P-S534L-V535A = Mut_{CT}. The chimera At_{NT}-PpSLAC2-Mut_{CT} behaved similar to the chimera At_{NT}-PpSLAC2-At_{CT} (Figures 4C and S2F), indicating that the conserved C-terminal motif is indeed necessary for functional SLAC1/OST1 interactions. It should be noted that this motif was not sufficient in itself; PpSLAC2-Mut_{CT} without the *Arabidopsis* N-terminal domain was not activated by the diverse OSTs (Figures 4C and S2G). This indicates that both the N terminus and C terminus together provide plant SLAC-type anion channels with the ability to be activated by ABA-dependent SnRK2 kinases. The results also suggest that the important C-terminal motif, which remained unaltered in SnRK2-regulated SLAC1-type anion channels from vascular plants, most likely first arose in a common ancestor of mosses (Figures 4D, S4A, and S4B).

DISCUSSION

Stomatal Guard Cells Use the Ancient ABA-Dependent SnR Kinase to Regulate Transpiration

Concerning the evolution of ABA-induced stomatal closure, the current opinions differ. On the one hand, lycophyte and fern stomata were shown to lack active responses to endogenous ABA, leading to the conclusion that the guard cell ABA-signaling system developed rather late in evolution of angiosperms, after the divergence of ferns [36, 37]. On the other hand, the targeted knockout of the *PpOST1-1* gene in *Physcomitrella patens* [32] and cross-species complementation studies have shown that the *Physcomitrella patens* SnR kinase PpOST1.1 is able to restore the ABA response in the stomata of AtOST1-deficient *Arabidopsis* plants [33, 38]. This suggested that the core regulatory components involved in guard cell ABA signaling of flowering plants are functional in mosses and likely originated in the LCA of these lineages [32, 33], prior to the evolution of ferns.

We have shown that SnRK2s from non-vascular plants can replace vascular plant OST1 in mediating ABA-dependent drought gene expression (Figure 2A) and fast guard cell anion channel activation (Figure 2B). Moreover, SnRK2s from all tested

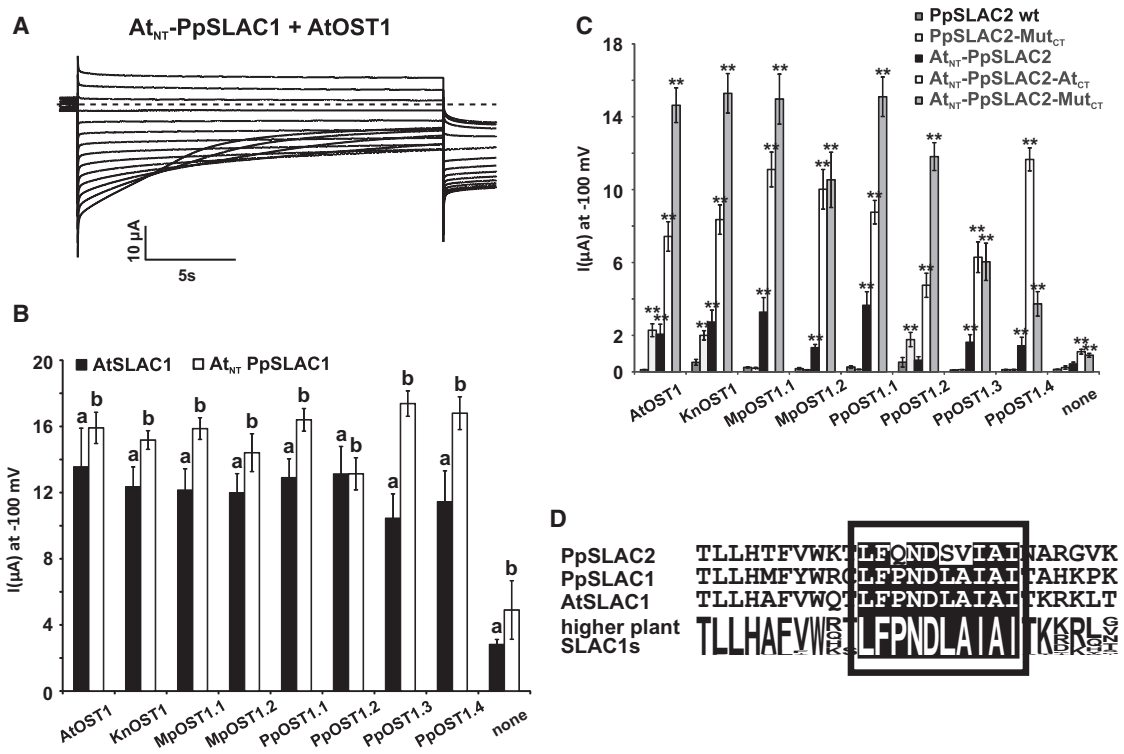

Figure 4. The AtSLAC1 N Terminus and C Terminus Render PpSLAC1 and PpSLAC2 Hybrid Proteins OST1 Sensitive

(A) Example whole-oocyte currents of At$_{NT}$-PpSLAC1 co-expressed with AtOST1 were recorded in a standard bath solution. Voltage pulses lasting 20 s ranging from +40 to −180 mV in 20-mV decrements were applied. The holding potential was clamped to 0 mV.

(B) Instantaneous currents (at −100 mV in standard solution) were recorded with either AtSLAC1 or At$_{NT}$-PpSLAC1 co-expressed alone (indicated as "none") or with OSTs from different species (mean ± SE; n ≥ 5 from experiments on at least two independent oocyte batches). In one-way ANOVA, we compared values obtained with AtSLAC1/kinase pairs with those obtained with AtSLAC1 alone (a) or values obtained with At$_{NT}$-PpSLAC1/kinase pair with those measured with At$_{NT}$-PpSLAC1 alone (b). Significant differences are labeled (a or b; $p \leq 0.01$).

(C) Instantaneous currents (at −100 mV in standard solution) recorded with either PpSLAC2 wild-type (WT) or different chimeras between PpSLAC2 and AtSLAC1 co-expressed with OSTs from different species. Data indicate mean ± SE (n ≥ 5 from measurements on at least two independent oocyte batches). In one-way ANOVA, we compared values of PpSLAC2 WT with those of chimeric or mutated anion channels; significant differences are indicated (**$p \leq 0.01$).

(D) Sequence comparison of the C-terminal OST1-interaction region. The ten-residue-long stretch LFPNDLAIAI (highlighted by a black frame) was 100% conserved in SLAC1 channels of vascular plants (shown as sequence logos in the lowermost line) and in PpSLAC1.

See Figure S4B for an extended sequence alignment.

species could be inhibited by co-expression of PP2C phosphatases (ABI1s; Figure 2C). Our studies further show that during land plant evolution, some SLAC proteins gained N- and C-terminal interaction sites that allowed SLAC1-expressing cells to co-opt the ancient OST1-dependent drought stress signaling for use in stomatal closure (Figures 4 and S4A). For such an OST1/SLAC1 module to be functional in cell-based valves that are able to inflate and deflate autonomously, another condition needs to be fulfilled: the module needs to be expressed specifically in guard cells; surrounding cells must not express the kinase-channel pair. This is known to be true in vascular plants like *Arabidopsis*, but further studies are needed to show exactly when in plant evolution fast ABA signaling via OST1 and SLAC1 became restricted to guard cells.

Several lines of evidence suggest a monophyly of stomata in the LCA of vascular plants, hornworts, and mosses (Figure 1A). The placement of liverworts is of particular importance in tracing the evolutionary origin of stomata. Current phylogenies either place them as the earliest diverging land plant lineage (Figure 1A and e.g., [7]) or cluster them together with mosses in a sister group to the remaining land plant lineages [39, 40]. While the first scenario is consistent with a monophyly of stomata after the divergence of liverworts, the latter or other evolutionary scenarios (e.g., [1]) suggest either convergent or parallel evolution of stomata in mosses, hornworts, and vascular plants or require secondary loss of stomata in the LCA of liverworts to explain the pattern of stomata occurrence.

The most straightforward interpretation of our results is that the innovation of stomata occurred in the LCA of mosses and vascular plants, based on its absence in *Marchantia* and *Klebsormidium* and on the assumption that liverworts diverged before the separation of mosses from vascular plants (Figure 1A). Should it turn out that liverworts group together with mosses, as suggested by some studies [39, 40], a scenario involving loss or independent evolution would appear necessary. Data from additional plant species will help to resolve this question in the future.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures.

ACCESSION NUMBERS

The European Nucleotide Archive accession numbers for the MpOST1.1, MpOST1.2, KnOST1, MpSLAC1, and KnSLAC1 coding sequences (cds) reported in this paper are LN794220, LN794221, LN794223, LN794219, and LN794222, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.067.

AUTHOR CONTRIBUTIONS

C.L., I.D., E.J.L.-S., K.v.M., K.I., D.L., and Y.Z. performed the experiments. C.L., I.D., K.I., T.K., D.L., Y.Z., I.K., and D.G. analyzed the data. I.D., K.A.S.A.-R., H.R., R.R., J.-K.Z., D.G., and R.H. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Tracey Ann Cuin for critical proofreading of the manuscript. We thank Eva L. Decker for her initial help in identifying the *Physcomitrella patens* OST and SLAC encoding gene models and Andreas D. Zimmer for his contributions in annotating the *K. nitens* genome and his initial phylogenetic analyses of the OST and SLAC proteins. This work was supported in parts by the Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS to R.R.) and by the NIH (Grant R01GM059138 to J.-K.Z.). H.R. was supported by grants from the Swedish Research Council VR (621-2011-6004) and the Swedish Strategic Research Foundation. I.D. was supported by a grant from the Spanish Ministerio de Economia y Competitividad (BFU2011-28815) and by a Marie Curie Career Integration Grant of the European Union (FP7-PEOPLE- 2011-CIG No. 303674–Regopoc). R.H., K.A.S.A.-R., and D.G. were supported by the King Abdullah Institute for Nanotechnology (KAINS), King Saud University, Riyadh, Saudi Arabia. R.H. and Ulrich Heber were further supported by the DFG grant HE 1640/23-1.

Received: October 10, 2014
Revised: January 14, 2015
Accepted: January 22, 2015
Published: March 19, 2015

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